

**Method for Producing L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine
and Derivatives Thereof**

The present invention relates to a method for producing a pharmaceutically active peptide compound, which contains L-m-sarcosine as the amino acid component. The active substance serves particularly for chemotherapy against cancers and is used especially for melanomas. In using a carrier substance on a cyclodextrin basis, the active substance is released delayed, which makes possible a sufficient bioavailability during a sufficiently long period of time.

10 A complex of six peptides containing m-L-sarcosine has become known under the trade name "Peptichemio" (Istituto Sieroterapico Milanese S. Belfanti, Milan, Italy) for chemotherapy against cancer. It has been found that the activity of the individual peptides is different and that particularly one representative exhibits very high toxicity to melanoma cells. The peptides are a development which began with the product "Melphalan," i.e., 4-[bis(2-chloroethyl)]-amino-L-phenylalanine. It has been found that this product has a cytostatic effect and can be utilized both for myeloma and for melanoma therapy. For the further development of the active substance, derivatives of the product were prepared. This also resulted in the L-m-sarcosine [= m-(di-2-chlorethyl)amino]-L-phenylalanine], which was further derived in that peptides were prepared which contained the modified amino acid as a component. A combination of the six oligopeptides L-seryl-L-p-fluorophenylalanyl-L-m-sarcosyl ethyl ester; L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester; L-m-sarcosyl-N-nitro-L-arginyl-L-norvaline ethyl ester; L-p-fluorophenylalanyl-L-m-sarcosyl-L-asparagine ethyl ester; <sic. L-p-> fluorophenylalanyl-glycyl-L-m-sarcosyl-norvaline ethyl ester and L-m-sarcosyl-L-arginyl-L-lysyl-L-m-sarcosyl-L-histidine methyl ester formed the active principle of the antitumor agent "Peptichemio." Of the six peptides, the L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine (PSF) and its lower alkyl esters have proven particularly suitable.

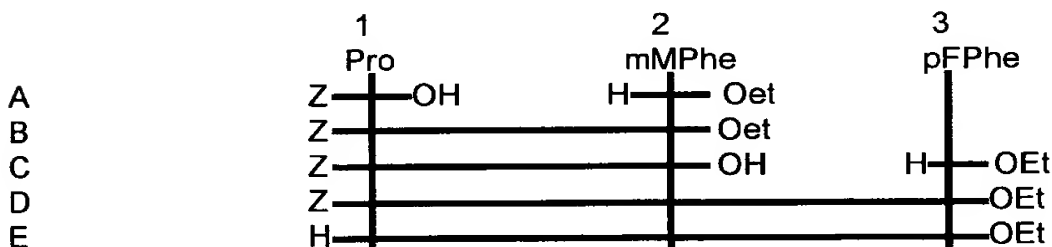
It has been found that PSF showed considerably higher cytotoxicity compared with peptichemio itself (R. Levenson, et al., Radiumhemmet, Karolinska Hospital, Stockholm, Sweden, Eur. J. Cancer Clin. Oncol.; 23: 6, 783-788, 1987). According to these studies, it has been found that the peptide

5 L-propyl-m-sarcolysyl-L-p-fluorophenylalanine (PSF) was 35 times and 28 times, respectively, more toxic to RPMI 8322 melanoma cells than melphalan and m-sarcolysine, respectively. Similar differences between the active substances have also been found for other melanoma cell lines.

It is the object of the present invention to make available a method of

10 producing PSF that makes possible an economical and safe production of the active substance.

Production of such a compound is described in the printed publications BE-A-775775 and US-A-3 814 746. The described production takes place according to the following schema 1:



15

Pro = proline
 mMPhe = m-[di(2-chlorethyl)]-amino-L-phenylalanine
 pFPhe = p-fluoro-L-phenylalanine
 Z = benzyloxycarbonyl

20 The above schema shows, in step A, the condensation of the N-carbobenzoxyl-L-proline with the ethyl ester of m-[di-(2-chlorethyl)-amino]-L-phenylalanine, the corresponding protected peptide resulting, as is shown in step B in schema 1.

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In step C, the N-carbobenzoxy-L-prolyl-m-[di-(2-chlorethyl)-amino]-L-phenylalanine is obtained from the N-carbobenzoxy-L-prolyl-m-[di-(2-chlorethyl)-amino]-L-phenylalanine ethyl ester, and subsequently the condensation of this compound is carried out with p-fluoro-L-phenylalanine ethyl ester, while in step D of the schema 1 the carbobenzoxy-L-prolyl-m-[di-(2-chlorethyl)amino]-L-phenylalanine-p-chloro-L-phenylalanine ethyl ester is obtained.

The protection group is eliminated, whereby one arrives at step E of schema 1, the end product being the L-prolyl-m-[di-(2-chlorethyl)amino]-L-alanine-p-fluoro-L-phenylalanine ethyl ester.

The reaction conditions are such as are used generally with peptide syntheses. With the above method, the end product is obtained with a yield of 30 %, with respect to the starting product m-[di-(2-chlorethyl)amino]-L-phenylalanine ethyl ester, it being necessary to carry out the purification of at least one intermediate product through column chromatography on silica gel.

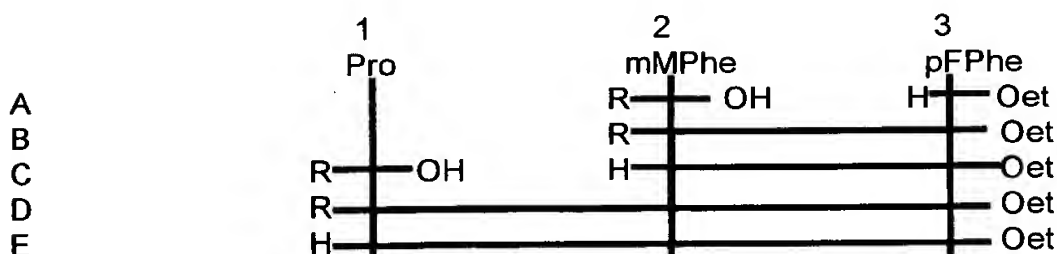
The method can actually be applied industrially, but it is relatively complicated and leads to a rather insufficient yield.

If one takes into consideration the properties of the end product PSF-hydrochloride, the realization of another method of production, which can easily be applied industrially and which results in a better yield than that of the state of the art, is then an extraordinarily important and interesting object of the present invention.

It has been found that the method for producing PSF according to the invention, which uses another reaction sequence, is superior to the state-of-the-art method.

The subject matter of the present invention is thus the method defined in claim 1 for producing L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine and esters and/or salts thereof.

The method according to the invention takes place according to the following schema 2:



Pro = proline

5 mMPhe = m-[di(2-chlorethyl)]-amino-L-phenylalanine (=L-m-sarcosine)

pFPhe = p-fluoro-L-phenylalanine

R = benzyloxycarbonyl, t-butoxycarbonyl (BOC) or
9-fluorenylmethoxycarbonyl (Fmoc)

10 The method comprises the following process steps, which are indicated in schema 2 above:

- a) condensation of R-m-[di-(2-chlorethyl)amino]-L-phenylalanine with p-fluoro-L-phenylalanine ethyl ester, whereby R-m-[di-2-chlorethyl)amino]-L-phenylalanyl-p-fluoro-L-phenylalanine ethyl ester is obtained;
- b) Removal of the protection group R ;
- 15 c) condensation of the product obtained in step b) with R-L-proline, R-L-prolyl-m-[di-(2-chlorethyl)amino]-L-phenylalanyl-p-fluoro-L-phenylalanine ethyl ester being obtained;
- d) Removal of the protection group R and synthesis of the hydrochloride;
R can be benzyloxycarbonyl, t-butyloxycarbonyl or 9-
20 fluorenylmethoxycabonyl. R is preferably a benzyloxycarbonyl group.

The method according to the present invention shows a total yield of 50 % with respect to the starting product R-m-[di-(2-chlorethyl)amino]-L-phenylalanine.

The method according to the present invention has a great advantage in carrying out the synthesis of the end product since crystalline intermediate products are obtained which can be purified extraordinarily easily through crystallization.

5 The features and the advantages of the method according to the invention will be explained, for better comprehension, in the following description. The tripeptide, produced according to the inventive method, is produced according to schema 2 above. Therein R is a benzyloxycarbonyl or a t-butoxycarbonyl group (BOC) or a 9-fluorenylmethoxycarbonyl group (Fmoc).

10 As follows from schema 2, the method calls for, in step A, the condensation of R-m-[di-2-chlorethyl)amino]-L-phenylalanine with the ethyl ester of the p-fluorophenylalanine, the corresponding protected tripeptide being obtained in step B.

15 In step C, the benzyloxycarbonyl group is removed, and through a condensation of the R-L-proline with m-[di-(2-chlorethyl)amino]-L-phenylalanyl-p-fluoro-L-phenylalanine ethyl ester, the R-L-prolyl-m-[di-2-chlorethyl)amino]-L-phenylalanyl-p-fluoro-L-phenylalanine ethyl ester is obtained in step D of schema 2.

20 The protection group R is removed in step E of schema 2, the end product being L-prolyl-m-[di-(2-chlorethyl)amino]-phenylalanyl-p-fluoro-phenylalanine ethyl ester.

The reaction conditions are such as are generally standard with peptide synthesis.

25 The peptide L-prolyl-m-sarcosyl-L-p-fluorophenylalanin (PSF) is produced preferably in the form of hydrochlorides or hydrobromides.

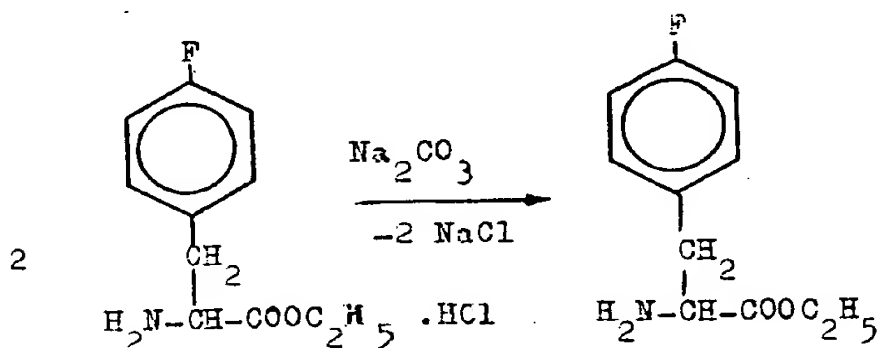
The following example serves the purpose of elucidation of the present invention.

Example:

Synthesis of L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester hydrochloride

a) N-carbobenzoxy-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester

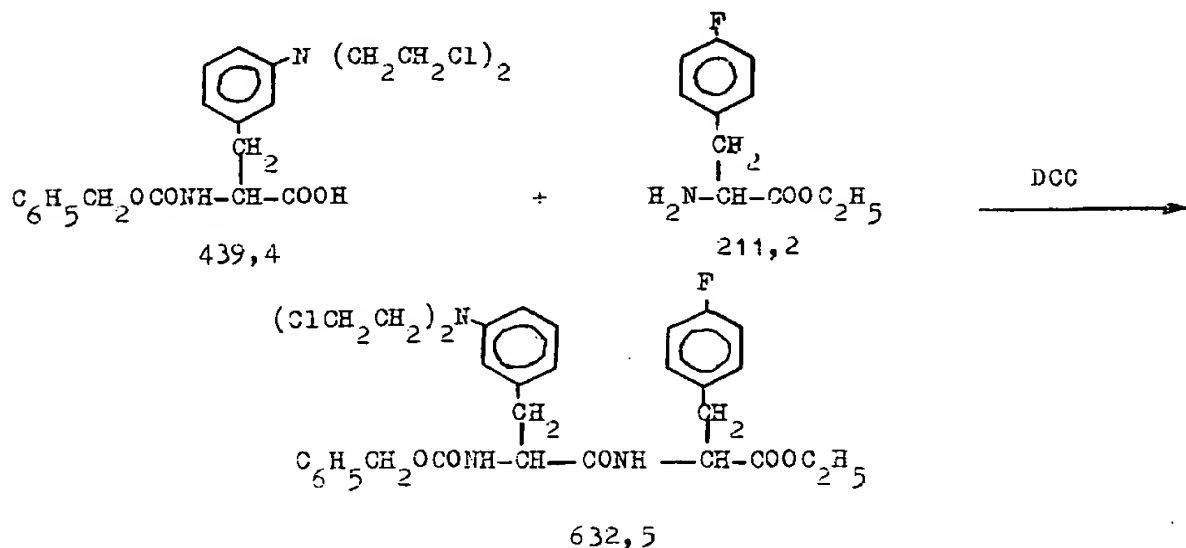
- 5 52.5 g of L-p-fluorophenylalanine ethyl ester hydrochloride are treated with 75 ml of Na_2CO_3 (sodium carbonate) saturated solution and 150 ml of CHCl_3 . The mixture is shaken out, and the organic phase is separated and saved. The aqueous phase is shaken out a second time with 75 ml of CHCl_3 . The combined chloroform extracts are mixed and washed once with water, and
- 10 then separated from the aqueous phase and dried on anhydrous Na_2SO_4 . The concentration of amino acid ester is determined by a titration with HClO_4 (perchloric acid). The yield corresponds approximately to the theoretical value; it is at 98%.



- 15 286.5 ml of a chloroform solution containing 0.1905 moles of L-p-fluorophenylalanine ethyl ester are reacted with 83.7 g (0.1905 moles) of N-cbzo-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester. The solution is cooled on an ice bath.

Added to the cooled solution with stirring are 41.25 g (0.200 moles of dicyclohexyl carbodiimide - DCC) and 60 ml of chloroform, the solution being

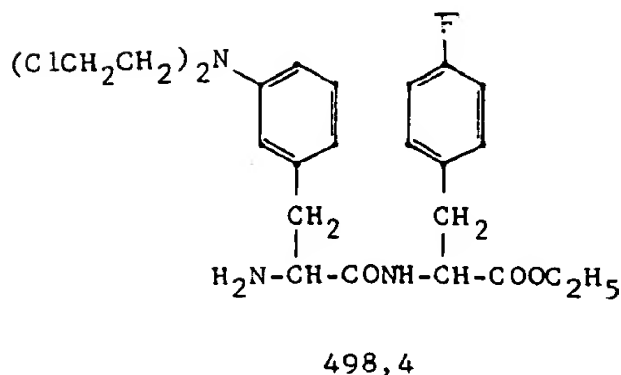
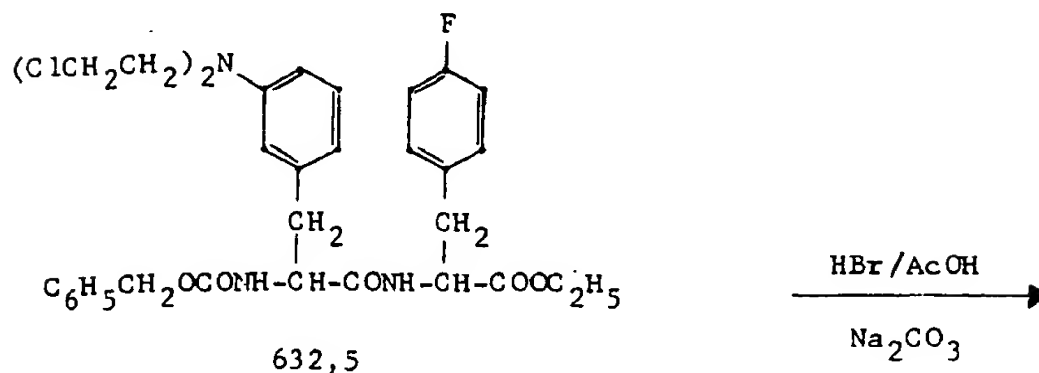
- constantly stirred with simultaneous cooling for 30 min. The mixture may possibly solidify into a solid mass. In this case, the mass is made liquid again through addition of 150 ml of chloroform, it being stirred with slight warming. In this way, dissolving of the precipitated product is accelerated. The reaction is
- 5 ended 2 hrs after addition of the DDC. The end of reaction is established by TLC checking (thin-layer chromatography; silica gel G layer, solvent: chloroform + acetone 9:1, manifestation by spraying with dilute, acid KMnO_4 solution). The precipitated dicyclohexyl urea is separated by filtration. The solution is washed first with little water, then with saturated Na_2CO_3 solution.
- 10 The chloroform solution is shaken out once more with water and then dried with Na_2SO_4 . The solvent is evaporated in vacuo and removed. After drying, 140.25 g of slightly yellowish-colored product is obtained (yield 98.3%). The substance produced has a melting point of 123-124.5°C and is chromatographically homogeneous. Through crystallization of 4.5 g of
- 15 substance from 37.5 ml ethyl alcohol, 3.75 g of a lighter product are produced with a melting point of 125-126°C. α_D^{20} : 27.7 ($c = 2$, CHCl_3).



Analysis for $\text{C}_{32}\text{H}_{36}\text{Cl}_2\text{FN}_3\text{O}_5$

N% = 6.67 (6.66 calculated)

Cl% = 11.5 (calculated = 11.2)

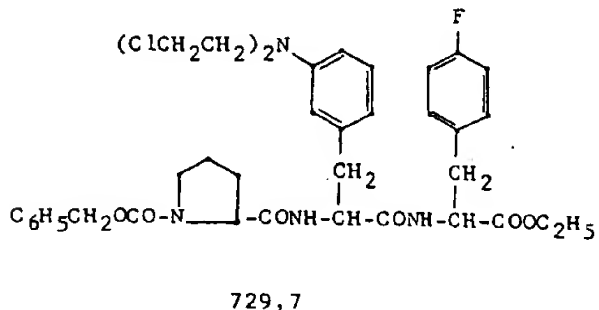
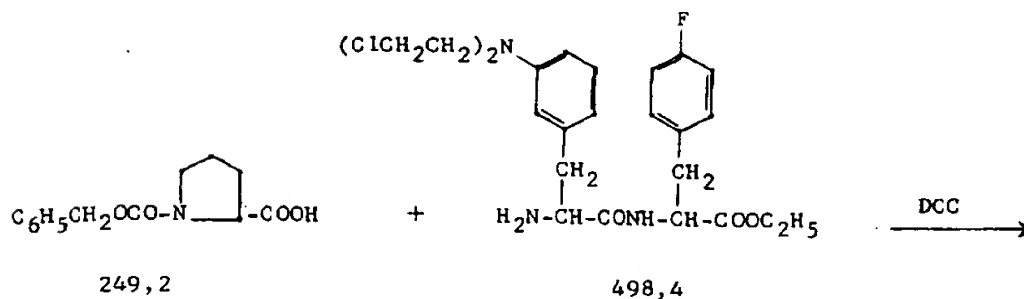
b) L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester

With exclusion of atmospheric humidity, 600 ml of HBr in glacial acetic acid (33%) are added with slow stirring to 390 g (0.616 moles) of die[?] M < sic. die[?] M N-> -carbobenzoxy-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester. Dissolving and cessation of the CO₂ development takes place after 40 minutes. It is allowed to stand for a further 20 minutes with stirring and diluted with approx. 400 ml of ether. The whole is poured into 5 lt of ether which is kept under constant stirring, is decanted, and the precipitated oil is washed twice with 2 lt of ether with decanting. The oil is treated with 4 lt of water with stirring, and a solid is obtained, which is collected after approx. 30 min. by filtration, and is completely washed with a total of 1500 ml of water and 500 ml of ether. The bromohydrate thus obtained is suspended in 2 lt of ethyl acetate and treated with stirring with 450 ml of saturated sodium carbonate solution, thus until the

solution is alkaline. After dissolving has taken place, filtration is carried out on the suction filter in order to remove the suspended dicyclohexyl urea (very little). In a separating funnel, the organic layer is separated from the aqueous phase, and the aqueous phase is extracted with a further 500 ml of ethyl acetate. The purified extracts are washed with 300 ml of water, Na_2CO_3 dried, and treated with norite. Filtration is carried out, and the filtrate is dried in vacuo (40°C). The residue is taken up even before it becomes firm in 500 to 1000 ml of ether. During the night, a white product is precipitated from the solution obtained. Yield: 247 g (80.4%) Melting point $100-102^\circ\text{C}$.

10 $\alpha_D^{20} = -7.5^\circ$ (c=2, chloroform)
 TLC (BuOH/AcOH/ H_2O 65:15:25; KMnO_4 diluted):
 one band, $R_f = 0,74$
 analysis for $\text{C}_{24}\text{H}_{30}\text{Cl}_2\text{FN}_3\text{O}_3$
 N% = 8.34 (8.43 calculated)
 15 Cl% = 14.1 (14.2 calculated)

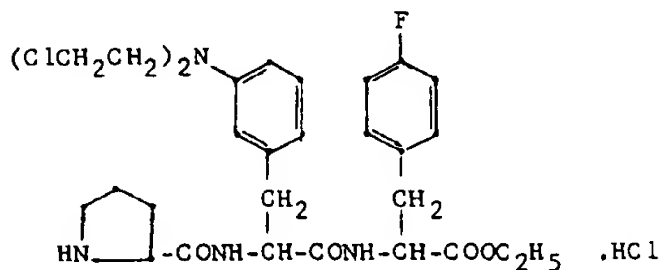
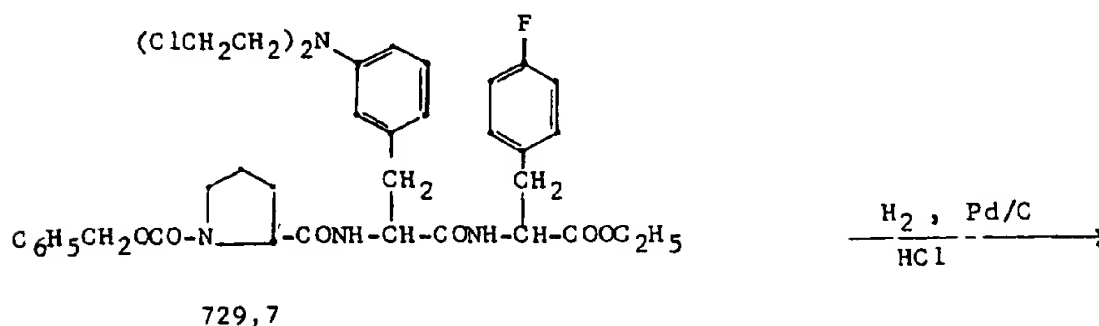
c) N-carbobenzoxy-L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester



- A mixture of 249 g (0.5 moles) of L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester, 125 g (0.5 moles) of N-cbzo-L-proline, and 109 g (0.525 moles) DCC in 3000 ml of chloroform is allowed to stand for 30 minutes with stirring, with external cooling for a further 90 minutes at room temperature (TLC, silica gel G, $\text{Chf}/\text{Me}_2\text{CO}$ 9:1; or with $\text{BuOH}/\text{AcOH}/\text{H}_2\text{O}$ 65:15:25; KMnO_4 , diluted, acid). After removal of the dicyclohexyl urea by filtration, the solvent is evaporated off in vacuo, and the residue, still in liquid state, is poured into 800 ml of ether. From the solution obtained, the product precipitates slowly out, which is collected on a filter. Yield 290 g (78.5%). Melting point = 148-150°C, $\alpha_D^{20} = -42.4^\circ$ (c=2; chloroform)

Analysis for $\text{C}_{37}\text{H}_{43}\text{FCl}_2\text{N}_4\text{O}_6$
 N% = 7.78% (7.68 calculated)
 Cl% = 9.6 (9.7 calculated)

- d) L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester
 15 hydrochloride



A mixture of 157.5 (0.261 moles) N-carbobenzoxy-L-prolyl-L-m-sarcosyl-L-p-fluorophenylalanine ethyl ester and 30 g of palladium on 5% carbon is suspended under a stream of nitrogen in 15 ml of glacial acetic acid and 1750 ml of methanol. The reaction mixture is kept stirred and is reduced
 5 under a stream of hydrogen. After termination of the CO₂ development (after 4-5 hours), a TLC chromatography check is carried out (silica gel G), elution taking place with chloroform acetone 9:1 and making visible with dilute KMnO₄.

After removal of the catalyst by filtration, the filtrate is acidified with concentrated ethanolic HCl in a stoichiometric amount or a little more. The
 10 white, crystalline precipitate which slowly forms is collected on a filter and washed with ethanol or with ether: 85 g. The filtrate is concentrated practically to dryness, and the residue is recrystallized from ethanol: 25 g. Complete yield: 110 g (80.5%); melting point 122-124°C (modification of the aggregate state)

$\alpha_D^{20} = 13.0^\circ \pm 0.5$ (c=2; MeOH)
 15 TLC (silica gel G; BuOH/AcOH/H₂O 65:15:25; KMnO₄ diluted: one band R_f = 0.54.
 Analysis for C₂₉H₃₈Cl₃FN₄O₄
 N % = 8.93% (8.86 calculated)
 Cl % = 16.7% (16.8 calculated)
 20 Cl-% = 5.65% (5.6 calculated)

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